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PATENT
Docket 1085-1

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Applicant:	Henri HANSSON et al.	Conf.:	3275
Appl. No.:	10/002,527	Group:	1619
Filed:	October 25, 2001	Examiner:	
For:	METHOD FOR THE PREDICTION OF STARCH DIGESTION		

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CLAIM TO PRIORITY

Assistant Commissioner for Patents
Washington, DC 20231

June 7, 2002

Sir:

Applicant(s) herewith claim(s) the benefit of the priority filing date of the following application(s) for the above-entitled U.S. application under the provisions of 35 U.S.C. § 119 and 37 C.F.R. § 1.55:

Country	Application No.	Filed
SWEDEN	0003876-0	October 25, 2000

Certified copy(ies) of the above-noted application(s) is(are) attached hereto.

Respectfully submitted,

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Attachment(s): 1 Certified Copy(ies)

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

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This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

PATENT- OCH REGISTRERINGSVERKET • PATENT- AND REGISTRATION OFFICE

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För Patent- och registreringsverket
For the Patent- and Registration Office

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Analytical method

The present invention concerns an analytical method for determining the resistance to degradation of native starch and for predicting – relying on *in vitro* tests - the speed of digestion of native starch in the gastrointestinal tract of mammals.

Background of the invention

Starch is an important source of carbohydrates and an ingredient in many food stuffs. When heat treated, e.g. boiled, the starch granules disintegrate and the starch is solubilised and gelatinised. For example cornstarch consists of granules sized 2 – 32 µm, mainly comprising two components, amylose and amylopectin. Amylose has a linear structure while amylopectin is branched. Both amylose and amylopectin consist of α-(1,4)-linked glucose residues while amylopectin also has α-(1,6)-linked glucose residues.

The starch granules are insoluble in cold water and swell in warm. The swelling is reversible until the temperature reaches about 55 to 65 °C. At this temperature the starch granules gelatinise and loose their crystalline structure. Gelatinised starch is then easily degraded by digestive enzymes present in the gastrointestinal tract, mainly by the action of α–amylase. In humans, α-amylase is present in the saliva and in the small intestine.

The digestibility of starch, both *in vivo* and *in vitro*, depends on the source of starch as well as its pre-treatment (e.g. native, fine / coarse, gelatinised or chemically modified). In the present description, claims and examples, the term "native starch" is used to define starch, that has not been subjected to heat-treatment or chemical treatment. The term "native starch" thus comprises both the vegetable and/or plant seeds, kernels or grains, as well as mechanically treated fractions, such as the milled and sieved product, granules and flour.

Native starch is however extremely slowly degraded in the human gastrointestinal tract. This is due to the three dimensional shape of the starch granules, making it difficult for the enzymes to access the carbon chains of the starch molecules.

Prior art

A method for analysing starch degradation has been disclosed by Robert L. Bruner (Determination of Reducing Value, in Methods in Carbohydrate Chemistry, 1964, pages 67 – 71) and has more or less remained the standard method since that time. The degradation of boiled starch by the action of α–amylase is measured as the concentration of free sugar in a starch suspension after the addition of an enzyme. Samples are taken at regular intervals and the reducing sugars, e.g. glucose and maltose, are reacted with a reagent, and the absorption determined photometrically, preferably spectrophotometrically. Enzymatic degradation is then plotted as sugar concentration as a function of incubation time. This method however has many drawbacks, e.g. poor repeatability and less than satisfactory accuracy.

Summary of the invention

The present inventors have surprisingly found that the degradation resistance of native starch is more accurately measured using an analytical method as disclosed in the attached claims. The inventive method is used to compare different types of starch, different fractions of starch or different starch formulations, and is used to predict the degradation resistance properties *in vivo*.

Short description of the drawings

The present invention will be disclosed in further detail in the following description and examples, and in the attached drawings, in which:

Fig. 1 shows the different release profile for filtered samples (solid line), compared to unfiltered samples (broken line);

Fig. 2 shows the release profile for untreated cornstarch with 0.01 M NaCl (solid line) and without NaCl (broken line) in the reaction buffer;

Fig. 3 shows the release profiles of different starch formulations, compared to untreated, native cornstarch, where the values for cornstarch encapsulated in ethyl cellulose (10 %) are marked with the symbol (O), cornstarch encapsulated in ethyl cellulose (20 %) with (△),

cornstarch encapsulated in guar gum (20 %) being marked with (✚) and the values for untreated cornstarch being marked (✖); and

Fig. 4 shows the correlation between the *in vitro* and *in vivo* behaviour of the formulations of Fig. 3, the symbols being the same.

Description

The present inventors have made available a novel method for the analysis of the degradation resistance properties of native starch, wherein a pre-determined amount of native starch is suspended in a buffer, a starch degrading substance added, and a reagent, forming a coloured complex with reducing sugars is added to a sample taken from the above mixture, followed by an evaluation or determination of the colour formed.. Preferably an enzyme, most preferably α–amylase, is used as the starch degrading substance. Preferably 3,5-dinitro salicylate is used as the reagent.

Specific features of the method include the following steps:

- a buffer is prepared, said buffer having about neutral pH or preferably a pH of about pH 6.6 and contains a small amount chloride ions, preferably about 0.01 M chloride ions;
- the test solution is incubated at about 37 °C;
- a sample is taken from the test solution and filtered before mixing with a reagent;
- the absorbancy is evaluated, preferably determined spectrophotometrically, and in particular measured by scanning the wavelength interval of 450 to 500 nm and the absorbancy determined at the maximum value occurring within this interval.

This method is preferably used to compare different fractions of native starch with respect to their ability to resist enzymatic degradation. The method can also be used to compare different starch formulations with respect to their ability to resist enzymatic degradation. Importantly, the inventive method simulates the natural digestion of starch in the gastrointestinal tract and the values obtained can be used to reliably predict the enzymatic degradation of starch *in vivo*.

According to the present invention, the reagent solution is prepared by dissolving 3,5-dinitro salicylate in aqueous NaOH. The reagent solution is stored in a dark place and filtered before

use. This has the advantage of removing precipitate, which easily forms in the reagent solution. According to an embodiment of the invention, the reagent solution is filtered at the time of addition of the reagent to the vessels, e.g. test tubes. According to an embodiment of the invention, the reagent solution if filtered through a 0.45 μm filter. The alkaline reagent solution has the advantage of terminating the enzymatic activity by denaturation of the enzyme.

Further according to the present invention, the buffer solution is prepared by dissolving KH_2PO_4 and NaOH in water, adjusting the pH to about neutral, preferably to about pH 6.6. NaCl is then added until the concentration of chloride ions is 0.01 M. The chloride ions have surprisingly been found to be very beneficial for the analysis, as they activate the enzyme and lead to a steeper release profile. This is an important advantage, as the time necessary for performing the analysis can be reduced. For the effect of the addition of NaCl, see Fig. 2.

The sample, a pre-determined amount of the starch to be analysed, is suspended in buffer, whereupon the vessels containing the sample-buffer suspension are placed in the incubation bath. According to the present invention, the samples are incubated at a temperature below the gelatinisation temperature of the starch, preferably at about 37 °C or preferably at 37 °C ± 0.5 °C. This not only simulates the temperature in the gastrointestinal tract more accurately than the prior art methods, conducted at room temperature, it also has the additional benefit of accelerating the enzymatic action and makes the determination less time consuming.

According to the inventive method, a sample is taken at time "zero", i.e. before addition of the enzyme solution. A "blank" or control sample is taken by mixing reagent and water. Further, a sample containing all the ingredients; buffer, reagent, enzyme and starch, is taken soon after addition of the starch, at about 1 to 20 minutes after addition of the enzyme. Preferably this sample is taken 2 – 10 minutes, and most preferably 5 minutes after addition of the enzyme. This called the "5 min sample" later in the attached Example. Further samples are taken at pre-determined intervals, e.g. at 10, 20, 30, and 45 minutes, at 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, and 4 h after addition of the enzyme. Obviously different intervals can be chosen, depending on the purpose of the study. Similarly, the study can be continued longer than the above 4 hours, e.g. 8, 10 or 12 hours or longer.

According to a preferred embodiment of the inventive method, also the sample aliquots are filtered before the evaluation or measurement of absorbancy. Preferably the samples are filtered though a 0.8 μm filter. For the effect of the filtration, see Fig. 1.

Importantly, each absorbance measurement is conducted in the form of a scan over the wavelength interval 450 – 500 nm, detecting the absorption maximum. The absorbance reading is then taken at this maximum. This constitutes a significant improvement over prior art measurements, e.g. analyses using a fixed measurement wavelength of 590 nm. The inventive method involving a scan, followed by measurement at the absorption maximum has lead to an improvement in accuracy and repeatability.

The wavelength interval has an additional advantage in that the α-amylase itself has an negligible absorption in this interval. In the filtered and reacted sample solution, the α-amylase absorbs light at the same wavelength as the reacted, reducing sugars. Thus, the main part of the measured absorbance at t = 5 min is due to the enzyme.

The degradation profile for native cornstarch granules is used as a standard. Depending on the purpose of the investigation, any starch fraction can be used as a standard or for comparative purposes. Fig. 3 illustrates a comparison, in that it shows the release profiles of three different starch compositions plotted in the same diagram, together with the release profile obtained for untreated cornstarch under the same conditions.

The advantages of the present invention include, but are not limited to, the easy handling and good repeatability and reliability of the analysis. Further, the inventive method provides an analytical method, well suited for practical studies of the digestibility of different types of starch, different fractions and/or qualities within the same species. Additionally, the inventive method constitutes a practical *in vitro* method, the results of which correlate closely with results obtained *in vivo* as seen in Fig. 4.

Example

A reagent was made by dissolving 3,5-dinitro salicylate (2.00 g, Aldrich) in aqueous NaOH (70 ml, 1 M). Optionally, the mixture is heated in order to expedite the formation of a clear solution. Upon cooling, water is added to 100 ml. The reagent solution is stored in a dark place and filtered through a 0.45 m filter before use, in order to remove possible precipitates.

The reagent solution was added in equal amounts (2 ml) in test tubes marked "control", "zero", "5 min", "10 min", "20 min", "30 min", "45 min", "1 h", "1.5 h", "2 h", "2.5 h", "3 h", "3.5 h", and "4 h". The test tubes were placed in an ice bath awaiting the analysis.

A buffer solution (pH 6.6) was made by mixing KH_2PO_4 (250.0 ml, 0.20 M, Sigma) and NaOH (89.0 ml, 0.20 M) and adding water to a total volume of 1000 ml. NaCl (0.58 g, Riedel-de Haën) was then added to produce a chloride concentration of 0.01 M.

A defined amount of starch to be investigated (in this experiment 4.0 g) is measured and suspended in the above buffer and placed in the degradation bath. The degradation bath is kept at a temperature of 37 °C ± 0.5 °C and stirred at a speed of 50 rpm. In the present example, 4.0 g untreated cornstarch (Maizena, Bestfood Nordic AB) was used.

According to an embodiment of the present invention, the enzymatic degradation properties of untreated or native starch granules of a known fraction are used for comparative purposes. Preferably native or untreated cornstarch granules of a known fraction are used.

An amount corresponding to 15 000 IU α–amylase (Type VI-B from porcine pancreas, Sigma) is measured and suspended in buffer. Before addition of the enzyme solution, a sample of the degradation bath is taken in order to determine the sugar concentration at "time zero". The sample is filtered through a 0.8 μm filter and an aliquot (2 ml) is pipetted to the test tube marked "zero". The same filter can be used throughout the series. The sample is boiled momentary (5 min) and placed in an ice-bath. Following this, the enzyme solution is added to the degradation bath and the time registered. Samples are then taken at predetermined intervals, such as the times indicated on the test tubes. The control is prepared by boiling reagent (2 ml) and water (2 ml) during 5 min and placing the sample in an ice-bath.

For each sample, the absorption is scanned in the interval 450 – 500 nm and the peak height registered for each absorption maximum. In order to determine the concentration of free

sugars (FS0) in the native starch, the absorbance of the sample "zero" is measured against a background of water and reagent, the control sample. Both samples and control are diluted by adding 11.6 ml water to 400 µl sample. The reacted and diluted sample solution is not stable (the reading falling 0.1 to 0.2 absorbancy units during 3 hours) so all samples are diluted slightly prior to the UV-spectrophotometric analysis.

In order to obtain a figure of the free, non-granulated starch present in the sample (and easily accessible for the enzyme), the "5 min" sample is analysed in the same manner as the control. This determination yields a measure of the amount of enzyme in the degradation bath, as the enzymes boiled with reagent absorb light at about the same wavelength as the reacted, reducing sugars. Following this, the "5 min" sample is used as background for the slower degradation of the native starch granules. This series of samples is diluted in the same manner as the control and "zero" sample, i.e. 11.6 ml water is added to 400 µl sample.

Optionally, also the "control" and the "zero" readings can be used for determination of the background and/or to obtain a complete degradation profile.

As the reacted samples loose stability when diluted, all samples should be diluted and analysed in sequence, unless the spectrophotometer has the ability to store a background scan. Undiluted samples are however stable during several hours.

The results of the spectrophotometric readings are then plotted as the absorbancy as a function of sample time. Alternatively a "sliding" average can be used. Using this later method, the closest previous and next coordinate (time, absorbation) is averaged, and the new point is introduced at the new co-ordinates. This way the scattering of the results in relation to the regression line is decreased and the changes in the equation of the line becomes negligibly small.

In Figure 1 it is seen how the regression of the release profile improves for filtered samples (solid line), compared to unfiltered samples (broken line). The samples were filtered through a 0.8 µm filter, the remaining experimental protocol being the same.

In Figure 2 it can be clearly seen how the presence of a small amount of chloride ions, in this case 0.01 M, results in a steeper release profile (solid line) than the profile plotted for the degradation in absence of chloride (broken line). The experimental procedure was as described above.

Figure 3 shows the release profiles for different starch formulations compared to native cornstarch (Maizena, Bestfood Nordic AB). In this figure, the values for cornstarch encapsulated in ethyl cellulose (10 %) are marked with the symbol (O), cornstarch encapsulated in ethyl cellulose (20 %) with (△), cornstarch encapsulated in guar gum (20 %) being marked with (✚) and the values for untreated cornstarch being marked (✖).

In Figure 4 the good correlation between the *in vitro* and *in vivo* behaviour of the above tested formulations is shown, the symbols being the same.

The *in vivo* behaviour was determined by measuring the blood glucose response according to the standard technique in 4 healthy, lean volunteers (age 35 to 45 years) with normal glucose tolerance. According to the "golden standard" of this technique, each substance was studied twice in each volunteer, and the mean value was calculated. The substances were tested in randomised order, at least one day apart. Moreover, the testing was performed under strictly standardized conditions. The subjects came to the laboratory in the morning, fasted for 10 hours. Physical activity was avoided right before and during the test. The test subjects were allowed to drink about 2 dl liquid, free from carbohydrates (water, tea or coffee) twice during the test; at 0 and 3 hours.

The capillary blood glucose level was determined in capillary blood samples (obtained by finger pricking) using a Glucometer DEX (Bayer Diagnostica AB) following the standard procedures for glucose measurements. At baseline, three consecutive blood glucose determinations were performed to ensure a stable baseline at time 0 hours. Thereafter the test substance (20.0 g) was ingested together with a standardized amount of water within 5 minutes. All liquids were carefully weighed and the same amounts ingested at each occasion to avoid variations in transit time through the gastrointestinal tract. The blood glucose determination was repeated at 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 hours.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention as set forth in the claims appended hereto.

Claims

1. Method for the analysis of the degradation resistance of native starch, wherein a pre-determined amount of native starch is suspended in a buffer, a starch degrading substance added, a reagent added, said reagent forming a coloured complex in the presence of reducing sugars, the components forming a test solution, **characterized** in that the test solution is incubated at a temperature below the gelatinisation temperature of the starch, and the color change evaluated, without preceding heat-treatment or chemical treatment of the starch.

2. Method according to claim 1, **characterized** in that

- the buffer has a pH of about neutral and contains a small amount of chloride ions,
- α–amylase is added to the starch suspension,
- the test solution is incubated at about 37 °C,
- a sample is taken from the test solution and filtered before mixing with the reagent, and
- the colour change of the test solution is determined as a function of time.

3. Method according to claim 1, **characterized** in that

- the buffer has a pH of about pH 6.6 and contains about 0.01 M chloride ions,
- α–amylase is added to the starch suspension,
- the test solution is incubated at 37 °C ± 0.5 °C,
- a sample is taken from the test solution and filtered before mixing with the reagent, and
- the absorbancy is measured by scanning the wavelength interval of 450 to 500 nm and the absorbancy determined at the maximum value occurring within this interval.

4. Method according to any one of claim 2 - 3 , **characterized** in that the reagent is 3,5-dinitro salicylate.

5. Method according to claim 4, **characterized** in that the reagent solution is filtered before use.

6. Method according to claim 3, **characterized** in that for each value, the average of two adjoining measurements is calculated, and the calculated value inserted at the co-ordinate in question.

7. Method according to claim 1, **characterized** in that the enzymatic degradation properties of untreated granules of a known fraction are used for comparative purposes.

8. Method according to claim 1, **characterized** in that different fractions of starch are compared with respect to their ability to resist enzymatic degradation.

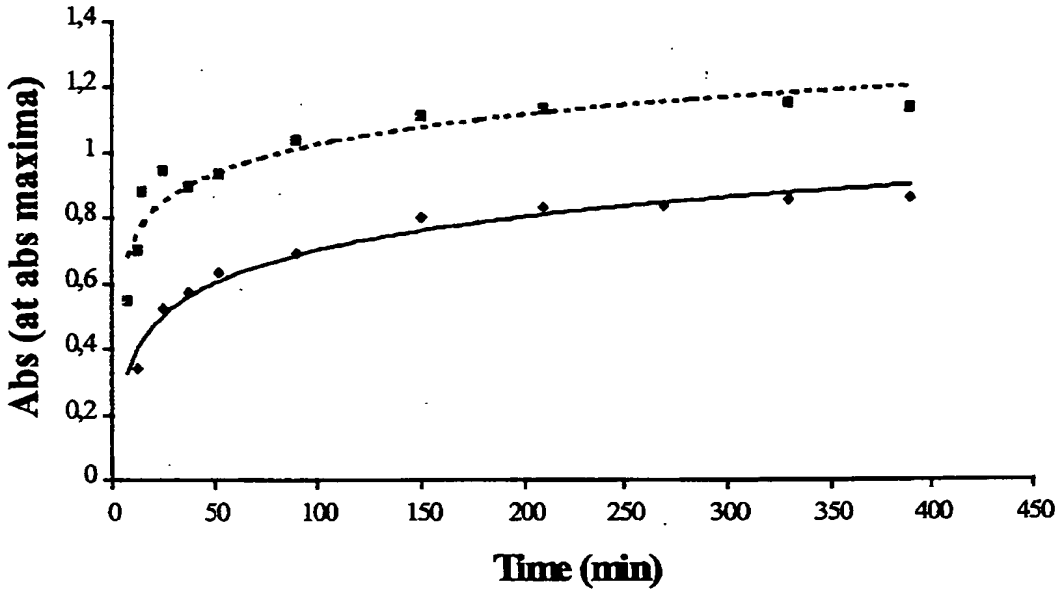
9. Method according to claim 1, **characterized** in that different starch formulations are compared with respect to their ability to resist enzymatic degradation.

10. Method according to any one of claims 1 through 9, **characterized** in that the values obtained are used to predict the enzymatic degradation of starch *in vivo*.

Abstract

The degradation resistance properties of native starch are studied *in vitro* using a photometrical method. The reducing sugars, formed by the enzymatic degradation of the starch molecules, form complexes with 3,5-dinitro salicylate, the concentration of which can be spectrophoto-metrically determined. The method has exhibited good accuracy and high reproducibility. The obtained results can be used to predict the enzymatic degradation behaviour *in vivo*.

(Fig. 4)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0
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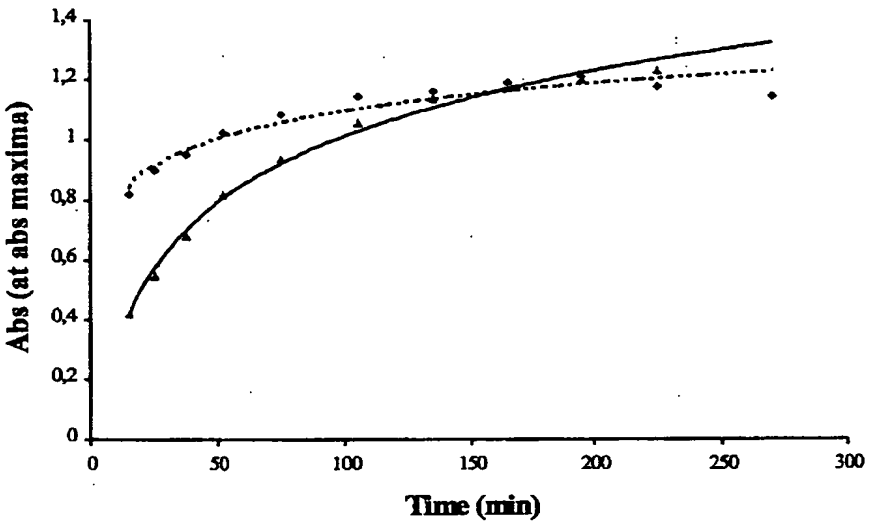
100
150
200
250
300
350
400
450
Time (min)

Fig. 1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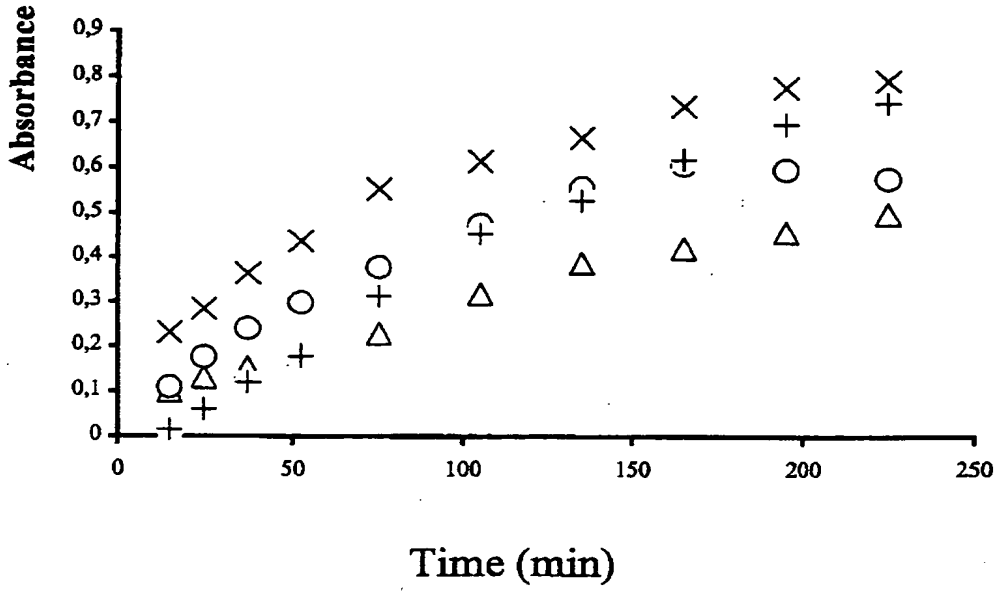
Time (min)

Fig. 2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0,6
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0,1
0
0
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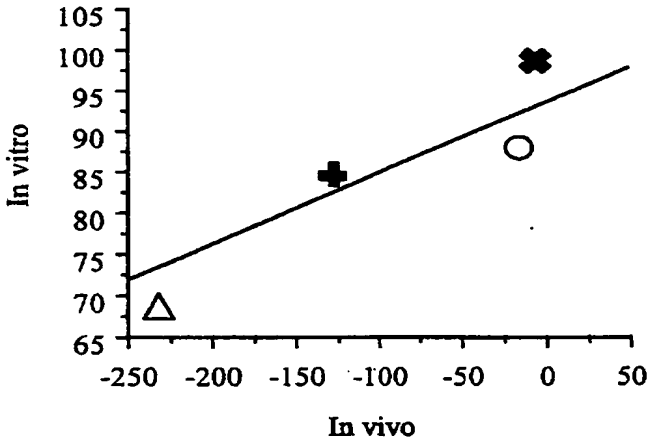
100
150
200
250
Time (min)

Fig. 3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80
75
70
65
In vitro
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-200
-150
-100
-50
0
50
In vivo

Fig. 4